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The Utility of Nuclear ITS, a *LEAFY* Homolog Intron, and Chloroplast *atpB-rbcL* Spacer Region Data in Phylogenetic Analyses and Species Delimitation in *Isoëtes*

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ABSTRACT.—Despite its ancient origins, its worldwide distribution, and adaptation to diverse habitats, *Isoëtes* has a highly conserved morphology. This feature has made it difficult to resolve species and species relationships using morphological characters. In this paper, we report the utility of nucleotide sequences from the nuclear internal transcribed spacer (ITS) regions, chloroplast *atpB/rbcL* intergenic spacer region, and second intron of a *LEAFY* (*LFY*) homolog for identifying species relationships, delimiting basic diploid species, and determining hybrid origins. Variation in the ITS regions and *atpB/rbcL* spacer is most useful at the family level in *Isoëtes* and the *LFY* second intron is appropriate at the species and population level. The tree resulting from an analysis of the combined nuclear ITS and chloroplast *atpB/rbcL* spacer contains three major well supported clades (bootstrap ≥ 99%): an Old-World/California clade (*I. abyssinica, I. longissima, I. velata, I. nuttallii,* and *I. orcuttii*), an Asian/Australian clade (*I. taiwanensis, I. japonica, I. kirkii,* and *I. drummondii*), and a poorly resolved clade consisting of nine North American species. To further resolve and delimit the North American species, a combination of the *LEAFY* second intron and ITS data was used. The resulting consensus tree has limited resolution, supporting the hypothesis that the North American species complex radiated rapidly. The combination of *LFY* and ITS data provided numerous characters, both substitutions and indels, that are useful in species delimitation and identification of cryptic species. ITS sequence data, through additive banding and sequence misalignment, is also useful in confirming interspecific hybrids and determining their parental origins.

Isoëtes is a cosmopolitan genus of heterosporous lycopsids containing 200 or more species. Despite the probable ancient Paleozoic origins of *Isoëtes,* its worldwide distribution, and its diverse ecological adaptations (from seasonal ephemeral terrestrials to obligate evergreen aquatics), *Isoëtes* species are singularly lacking in morphological variation (Taylor and Hickey 1992). The taxonomic difficuties caused by conserved morphology are compounded by homoplasy and allopolyploid speciation. Because of these problems, *Isoëtes* is a prime candidate for additional sources of data, such as DNA sequences.

A typical *Isoëtes* sporophyte consists of a buried rootstock bearing a rosette of simple, linear sporophylls. Each sporophyll contains four longitudinal, septate canals and an ovoid sporangium near its base. Each sporangium contains either scores of globose megaspores or thousands of ellipsoid microspores. A flap of tissue, called the velum, partly or completely covers the sporangium. Above the sporangium is a triangular ligule.

Since the Paleozoic Era, there have been numerous opportunities for species

of *Isoëtes* to adapt repeatedly to terrestrial habitats during drier conditions and to aquatic habitats in wetter periods. The range of habitats in which the species are found today appears to be a reflection of these past adaptations. For example, some species are evergreen aquatics in glacial lakes. Other species are ephemeral, seasonal terrestrials in soil pockets on exposed rock outcrops. Still others are amphibious, found in and along intermittent streams. A few species invade old fields and roadside ditches. *Isoëtes* appears to be an opportunistic pioneer able to adapt to a wide range of habitats.

Repeated adaptations to aquatic and terrestrial environments over time could lead to reversals, parallelisms, and convergences in morphological data sets. Thus it is possible that homoplasious morphological character states have been used to identify and relate species. A phylogeny of *Isoëtes,* based on few, potentially homoplasious character states, would probably be an inaccurate reconstruction. Therefore, alternative data sets are critical for reconstructing phylogenies and defining species within *Isoëtes.*

Chromosome counts have provided some insight into the evolutionary history of *Isoëtes.* For example, about one-half of the North American species are basic diploids (2n = 22, × = 11). The other half form a polyploid series of tetraploid (2n = 44), hexaploid (2n = 66), octaploid (2n = 88), and decaploid (2n = 110) species. These chromosome numbers indicate that *Isoëtes* has evolved in two ways. Basic diploid species have evolved by ecological isolation and genetic divergence as taxa adapted to terrestrial and aquatic habitats. Polyploid species have evolved through interspecific hybridization and chromosome doubling when divergent species occupied overlapping ranges. Interspecific hybrids, recognized by their irregularly formed and nonviable megaspores, are commonly encountered where two or more species occur together. At least fifteen different interspecific hybrids of *Isoëtes* have been detected among North American species (Taylor *et al.,* 1993; Montgomery and Taylor, 1994; Britton and Brunton, 1995, 1996; Musselman *et al.*, 1995, 1996, 1997). Interspecific hybrids and putative allopolyploids have caused confusion in defining species because they possess character states intermediate between the two parental species.

The main purpose of this paper is to show the usefulness of sequence data for ITS, the *atpB/rbcL* spacer region, and especially the second intron of the *LEAFY* (*LFY*) ortholog, to address phylogenetic questions. Future work will result in more extensive data (including many more species sampled) for resolving phylogenetic relationships and questions related to biogeography, species delimitation, and hybrid origins.

Variation in the ITS regions and *atpB/rbcL* spacer is most useful at the family level in *Isoëtes* and the *LFY* second intron is appropriate at the species and population level. The two ITS regions are located between the 18S and 26S nuclear ribosomal genes and separated by the 5.8S gene. There are multiple copies of this ribosomal array in the genome, but they appear to undergo rapid concerted evolution and all copies appear to be virtually identical in *Isoëtes* (Wendel *et al.*, 1995; Baldwin *et al.*, 1995; our data). The two ITS regions together are about 590 bp in length in *Isoëtes* but nucleotide substitutions and

insertions/deletions (indels) are common. ITS regions have been used extensively to study angiosperm taxa at the species and generic level (Baldwin *et al.*, 1995).

The *atpB/rbcL* intergenic spacer is located between two highly conserved chloroplast genes, *atpB* and *rbcL*. This spacer region is approximately 750 bp long, but indels are common. Spacer sequences have been used in many phylogenetic studies (e.g., Golenberg *et al.*, 1993; Manen *et al.*, 1994; Hoot and Douglas, 1998).

LFY is a meristem identity gene that encodes a transcription factor involved in the activation of homeotic genes which control flowering in *Arabidopsis* (Parcy *et al.*, 1998). *LFY* homologs have been identified in *Pinus, Picea, Welwitschia, Gnetum,* ferns, *Huperzia, Lycopodium,* and *Selaginella* (Frohlich and Meyerowitz, 1997; Mouradov *et al.*, 1998; Frohlich, pers. comm.; Therrien, pers. comm.). *LFY* is usually found in one copy in higher plants but *Isoëtes* has two copies. These two copies, including short stretches of the flanking exons, differ in length by about 100 bp (long copy ca. 1150 bp; short copy ca. 1050 bp). The partial exon sequences of the two copies are highly conserved and readily aligned, but the two introns have very different nucleotide sequences. For our work on *Isoëtes,* only the long copy of intron 2 was sequenced.

Materials and Methods

Total DNA was isolated from 0.3–2.0 g of fresh or silica-dried leaf tissue (Table 1) following the CTAB extraction procedure of Doyle & Doyle (1987).

The ITS regions, including the 5.8S gene, were amplified by the polymerase chain reaction using primers 1830F, located in the 18S gene, and 25R, located in the 26S gene (for primer sequences, see Nickrent *et al.*, 1994). Double-stranded amplifications in 100 μL reactions were conducted with the following reagents: 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$,10 M DMSO, 50 μM each dNTP, 0.25 μM of each amplification primer, and 2.5 U Taq polymerase. After overlaying each reaction with mineral oil, genomic DNA (210–500 ng) was added. Reaction conditions consisted of 40 cycles of 94° C for 30 sec, 45°C for 30 sec, and 72°C for 2 min for the denaturation, annealing, and extension steps, respectively. The first cycle was preceeded by a 4 min denaturation step and the last cycle by a 5 min extension step. The *atpB-rbcL* intergenic spacer region was amplified as described in Hoot and Douglas (1995) using primers located within the *atpB* and *rbcL* genes for both amplification and sequencing.

The second intron of *LFY* (long copy) was amplified using the degenerate primers LFsxl-3 and LFtxr (Frohlich and Meyerowitz, 1997). For five species, the two copies of *LFY* present in *Isoëtes* were substantially different in size and could be separated on a 1.5% agarose gel overnight. The two bands were then excised, reamplified using the melted gel plug as template, and used for sequencing. The resulting sequences were then aligned to locate a conserved region within the second intron that could be used to design internal ampli-

TABLE 1. Collection data for species analyzed.

Isoëtes	*Country/State*	*Collector*	*Collection*
I. abyssinica Chiov.	Kenya	Gastony	97–101
I. butleri Engelm.	USA/Kansas	McGregor	*s.n.*
I. coromandelina L.	India	Srivastave	*s.n.*
I. drummondii A. Br.	Australia	Hoot	*s.n.*
I. echinospora Dur.	USA/Maine	Taylor	5137
I. echinospora Dur.	USA/Montana	Taylor	*s.n.*
I. echinospora Dur.	USA/Wisconsin	Taylor	5097
I. engelmannii A. Br.	USA/Michigan	Taylor	*s.n.*
I. Engelmannii A. Br.	USA/Missouri	Taylor	*s.n.*
I. Engelmannii A. Br.	USA/Virginia	Taylor	6015
I. flaccida Shuttlew.	USA/Florida	Taylor	5214
I. hawaiiensis Taylor & Wagner	USA/Hawaii	Taylor	5658
I. japonica A. Br.	Japan	Takahashi	*s.n.*
I. kirkii A. Br.	New Zealand	Woodland	*s.n.*
I. lithophila Pfeiffer	USA/Texas	Taylor	4653
I. longissima Bory	Spain	Taylor	5409
I. melanopoda Gay & Dur.	USA/Louisiana	Lark	*s.n.*
I. melanopoda Gay & Dur.	USA/Mississippi	Leonard	*s.n.*
I. melanopoda Gay & Dur.	USA/Arkansas	Johnson	*s.n.*
I. melanospora Engelm.	USA/Georgia	Taylor	4849
I. nuttallii A. Br.	USA/California	Taylor	5446
I. orcuttii A. A. Eat.	USA/California	Taylor	5447
I. prototypus Britton	Canada	Brunton	12350
I. taiwanensis DeVol	Taiwan	Kuo	*s.n.*
I. tegetiformans Rury	USA/Georgia	Taylor	5182
I. valida (A.A. Eat.) Clute	USA/North Carolina	Taylor	*s.n.*
I. velata A. Br.	Spain	Prada	*s.n.*

fication primers. These internal primers were specific to just the long copy of the *LFY* intron and are located approximately half way through the intron. The long copy of the *LFY* second intron was then amplified in two parts. Amplification followed the same protocol described above for ITS except that an initial 25 μl amplification reaction was done with Ready-To-Go Beads (Amersham Pharmacia Biotech) and the resulting PCR product used as the template for future reactions.

Following amplification, double-stranded PCR product was freed of remaining nucleotides and primers by electrophoresis on a low melting 2% agarose gel. The bands were visualized with UV light source, removed as gel plugs, and melted at 70°C. DNA was further purified and concentrated using Wizard Columns (Promega), following the manufacturer's protocols. Purified PCR products were sequenced on an ABI 373-Stretch automated sequencer. The two amplification primers were used for each region with additional internal sequencing primers employed for the ITS and *atpB-rbcL* spacer regions (primer sequences available upon request from S. Hoot).

Alignment of DNA sequences was accomplished to a rough approximation using Sequencher 3.0 (Gene Codes Corp.) with subsequent manual corrections. The criteria for indel alignment and scoring are as described in Hoot and Doug-

las (1998). Phylogenetically informative indels (variable in two or more taxa) were scored as one event at the end of the data set.

Fitch parsimony of the separate and combined data sets were performed with PAUP* vers.4.0b2a (Swofford, 1998) using the branch and bound search option. To assess branch support, PAUP* was used to perform bootstrap analyses (Felsenstein, 1985) with 500 replications, the branch and bound search option, and maximum number of trees saved set at 2000.

Isoëtes is morphologically very distinct and does not appear to be closely related to any extant pteridophytes. We are currently working on rooting our molecular trees using various genera within the Lycopodiaceae, the nearest sister group to *Isoëtes* (Manhart, 1994; Wikström and Kenrick 1997). As this work is not yet complete, we chose *I. coromandelina* L. f. as the root of our tree based on three lines of evidence: 1) preliminary *rbcL* data for selected species of *Isoëtes* and lycopods, 2) previous morphological work suggesting that the more plesiomorphic character states and geographic distributions are found in section *Coromandelina* (Taylor & Hickey, 1992), and 3) molecular evolution in *Isoëtes* appears to evolve in a clocklike fashion, suggesting that the root of the tree will be among the species with the greatest number of substitutions (this work and unpublished preliminary data). For the analysis of the combined LFY/ITS data (North American species complex), the tree was rooted using the ITS sequence for *I. drummondii* A. Br., based on the results of our worldwide phylogeny (Fig. 1). Due to sequencing difficulties, a sequence for *I. drummondii* was not available for *LFY*; the data for that region is scored as missing for this species in the combined data set.

Results

Relationships of Basic Diploid Species.—A combination of ITS and *atpB-rbcL* spacer data was used to evaluate relationships on a global scale and resulted in seven most parsimonious trees based on 176 informative characters (312 variable characters) with a consistency index excluding uninformative characters (CI) = 0.79 and retention index (RI) = 0.90. One of the seven shortest trees is presented in Fig. 1.

Three major well supported clades (bootstrap $\geq$ 99%) are recognized: an Old-World/California clade (*I. nuttalii, I. orcuttii, I. abyssinica, I. longissima,* and *I. velata*), an Asian clade (*I. kirkii, I.drummondii, I. taiwanensis,* and *I. japonica*), and a North American clade (*I. flaccida, I. prototypus, I. melanopoda, I. lithophila, I. melanospora, I. engelmannii, I. valida, I. echinospora,* and *I. hawaiiensis*). The North American species are not monophyletic. *Isoëtes nuttallii* and *I. orcuttii* from California are nested within a clade consisting of Spanish and African species, separate from the remaining North American species. The North American clade, referred to in the future as the North American species complex, is poorly resolved, suggesting a possible rapid radiation event with subsequent speciation.

Species relationships and delimitation within the North American species complex.—To resolve and delimit species within the North American spe-

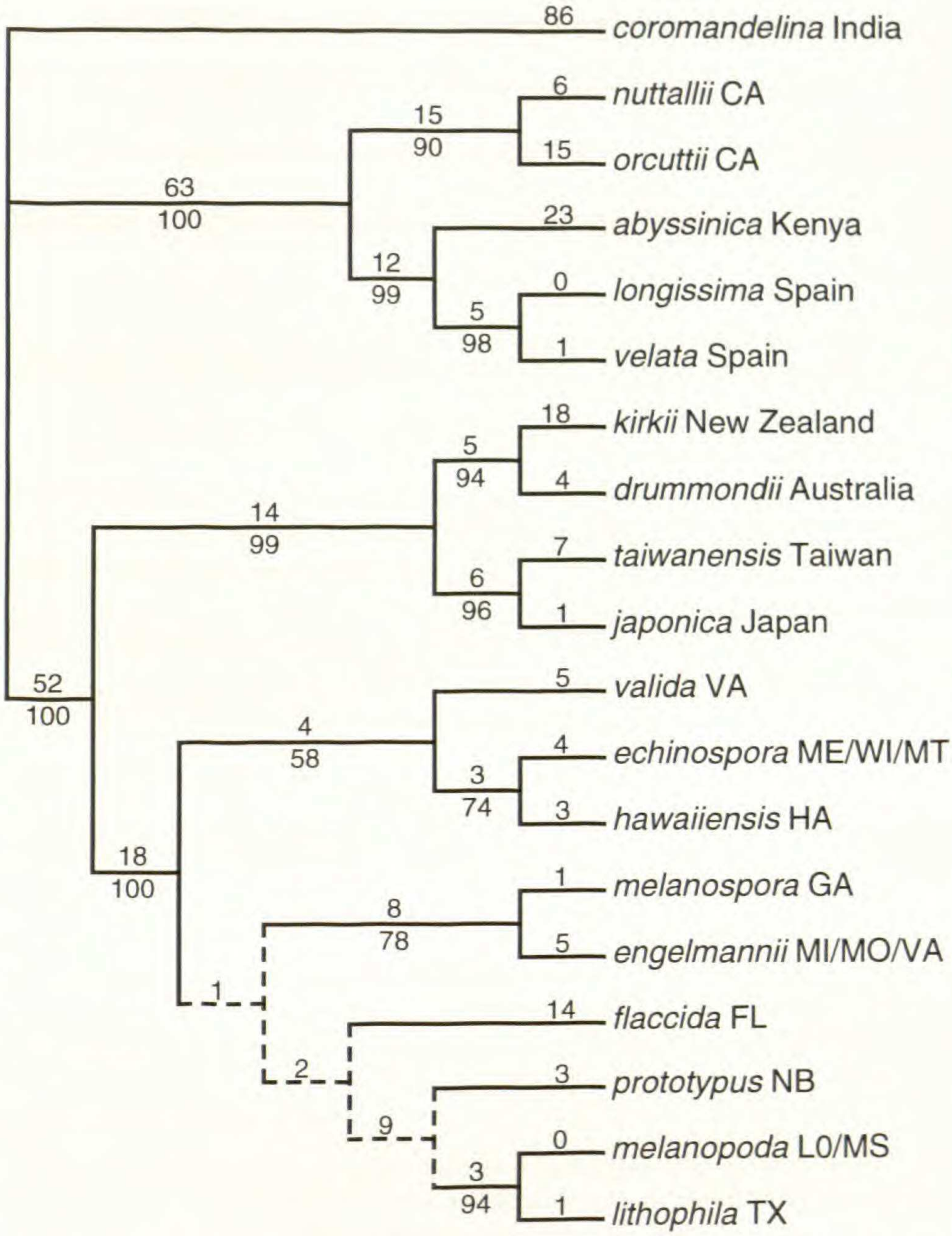

FIG. 1. One of the seven most parsimonious trees derived from the combined ITS and *atpB-rbcL* spacer region data. Numerals above the lines indicate the number of nucleotide changes supporting each branch (using accelerated transformation optimization); numbers below branches are bootstrap values. Dotted lines indicate branches that collapse in the strict consensus tree derived from multiple shortest trees.

cies complex, a combination of ITS and *LFY* intron data was used. The *LFY* intron data, consisting of 218 variable characters and 24 indels, is more than four times more variable than ITS (48 variable characters and four indels). The eight trees resulting from the analysis of *LFY* intron data alone (tree not presented) are largely similar to the combined *LFY*/ITS tree (Fig. 2), differing only in the position of *I. butleri.* (sister to *I. engelmannii* in the *LFY* tree). There was no resolution of the North American species complex using ITS data alone.

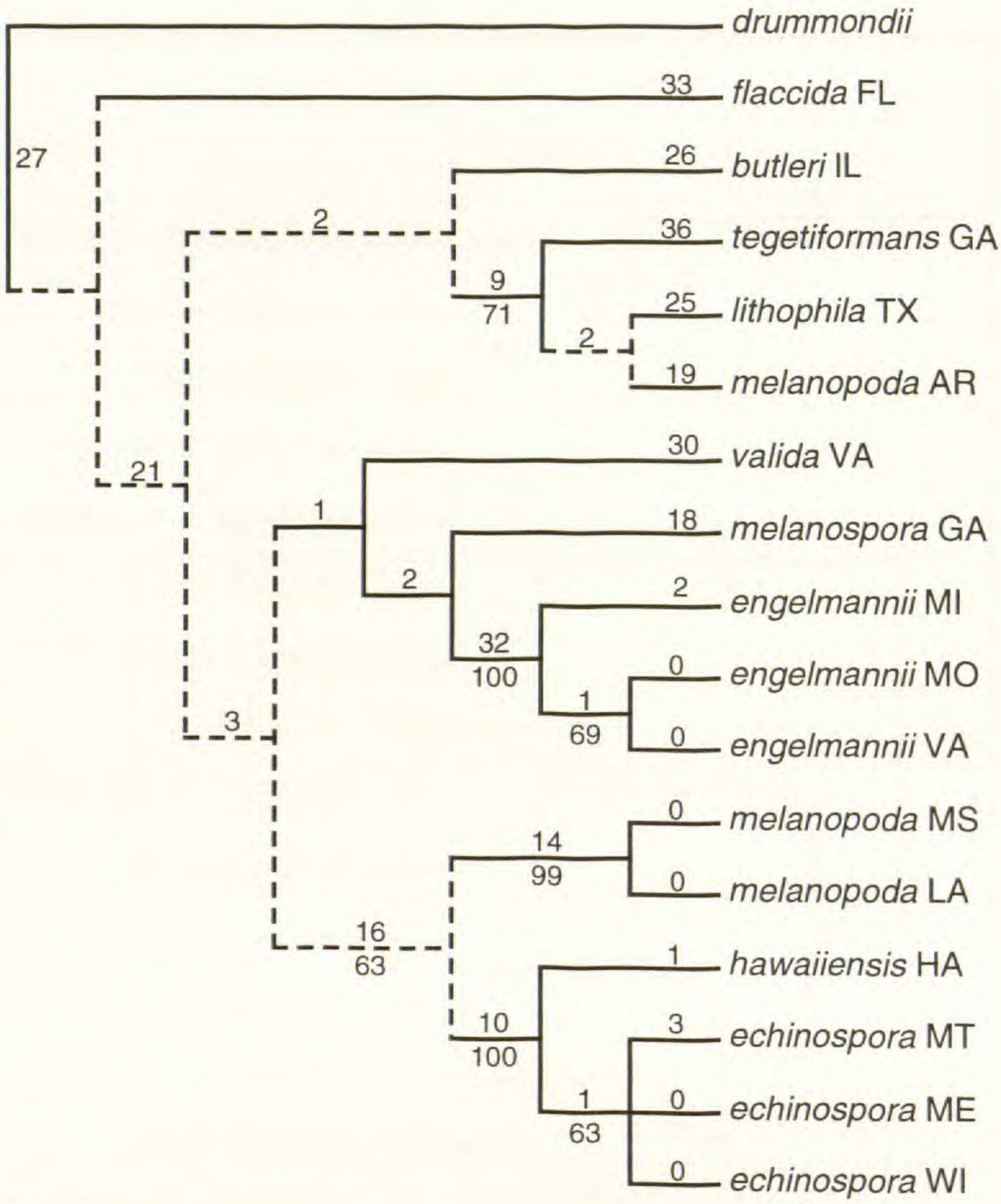

FIG. 2. One of six most parsimonious trees derived from the combined *LFY* intron 2 and ITS data. Numerals above the lines indicate the number of nucleotide changes supporting each branch (using accelerated transformation optimization); numbers below branches are bootstrap values. Dotted lines indicate branches that collapse in the strict consensus tree derived from multiple shortest trees.

One of the seven shortest trees (94 informative characters, CI = 0.77 and RI = 0.88) resulting from the analysis of the combined ITS and *LFY* intron data is presented in Fig. 2. The deeper branches of the tree collapse in the strict consensus tree, supporting the hypotheses that the North American species complex radiated rapidly.

Within the North American species complex, there is some resolution of species relationships (Fig. 2). *Isoëtes tegetiformans*, *I. lithophila*, and *I. melanopoda* (population from Arkansas) form a moderately well supported clade (9 substitutions, bootstrap = 71%). Unlike the results from a combination of ITS and spacer data, *I. melanopoda* populations from Louisiana and Mississippi

are weakly supported (bootstrap = 63%) as sister to a well supported clade consisting of *I. hawaiiensis* and *I. echinospora* (bootstrap = 100%). While the position of *I. valida* changes between the two trees derived from ITS/spacer and *LFY*/ITS, none of the relevant branches are well-supported (bootstrap ≤ 58%).

The combination of *LFY* and ITS data provide numerous characters, both substitutions and indels, which are useful in delimiting species (Fig. 2). Plants from three widely separated populations of *I. engelmannii* from Michigan, Missouri, and Virginia occur in the same well-supported clade (Fig. 2; 32 characters, bootstrap = 100%). Similarly, individuals from widely separated populations of *I. echinospora* from Maine, Wisconsin, and Montana, are nearly identical, only differing from each other by three unique sites found in the Montana population.

In contrast, the tree resulting from the combined *LFY* and ITS data indicates that *I. melanopoda* as currently defined is polyphyletic and composed of at least two species. *Isoëtes melanopoda* from Arkansas, differs from two populations of *I. melanopoda* from Louisiana and Mississippi by numerous substitution and indels. The former appears in a moderately-supported clade with *I. lithophila* and *I. tegetiformans* (bootstrap = 71%).

DETERMINING THE ORIGIN OF ALLOPOLYPLOID SPECIES.—We used nucleotide sequences of the ITS regions of nuclear ribosomal DNA to tentatively determine the origins of the interspecific hybrid *I.* ×*eatonii* Dodge and the allotetraploid *I. riparia* Engelm. ex A. Br. Taylor and Hickey (1992) provided data from distribution patterns, spore morphology and viability, electrophoretic profiles of leaf enzymes, and in vitro hybridization experiments supporting the hypothesis that the basic diploid species *I. echinospora* (2n = 22) and *I. engelmannii* (2n = 22) have crossed to form the sterile hybrid *I.* ×*eatonii* (2n = 22) and that chromosome doubling has produced the fertile allotetraploid, *I. riparia* (2n = 44).

When ITS sequences of *I. echinospora, I. engelmannii,* their hybrid, *I.* ×*eatonii,* and *I. riparia* are aligned and compared, nucleotide additivity and misalignment are apparent in the sequence chromatograms (Fig. 3). Nucleotide additivity occurs in *I.* ×*eatonii* and *I. riparia* at nucleotide positions where *I. echinospora* and *I. engelmannii* differ. Nucleotide misalignment occurs in *I.* ×*eatonii* and *I. riparia* in the 5′ to 3′ direction (determined by the direction of primer extension) where there is a nucleotide insertion or deletion in either *I. echinospor*a or *I. engelmannii* (Fig. 3). ITS nucleotide additivity occurs at each of 10 sites in *I.* ×*eatonii* and at 8 sites in *I. riparia* where *I. echinospora* and *I. engelmannii* differ (Table 2). There are also two indels in *I. echinospora* and *engelmannii,* leading to nucleotide misalignments (beginning at sites 49 and 548) in *I.* ×*eatonii* and *I. riparia.*

DISCUSSION

RELATIONSHIPS OF BASIC DIPLOID SPECIES.—The combination of nucleotide sequences from ITS and the *atpB/rbcL* intergenic spacer are effective in resolving

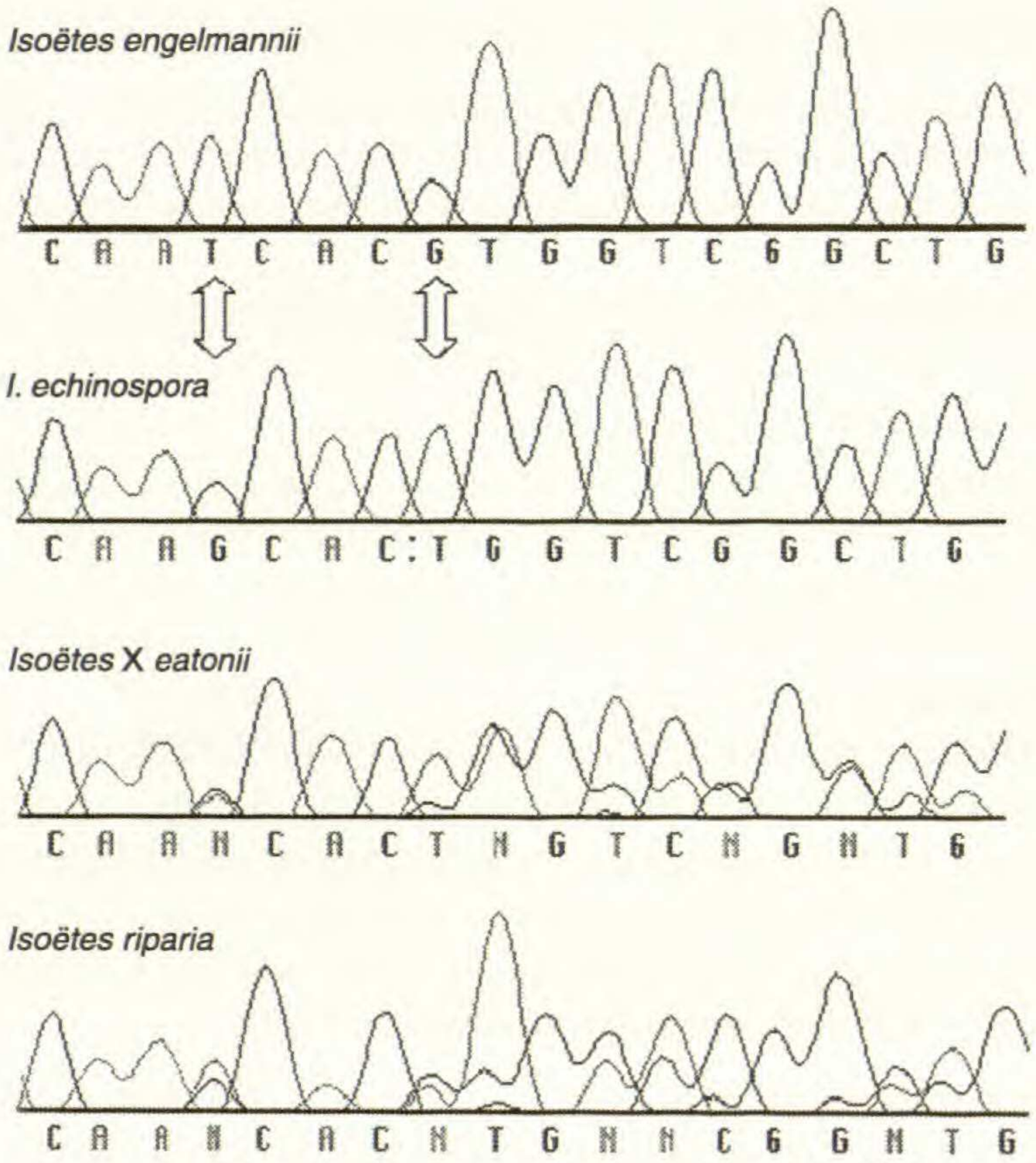

FIG. 3. Aligned sequence chromatograms of *I. engelmannii*, *I. echinospora*, *I. ×eatonii*, and *I. riparia* for an 18 bp segment in the ITS2 region. The arrow on the left indicates site 544 where the two parental species, *I. engelmannii* and *I. echinospora*, differ in nucleotides and additive banding is found in the two taxa of hybrid origin, *I. ×eatonii*, and *I. riparia*. The arrow on the right indicates the location of a deletion in *I. echinospora* in relation to *I. engelmannii*. The two parental sequences found in *I. ×eatonii* and *I. riparia* are misaligned to the right of this deletion in the direction of the primer extension.

phylogenetic relationships of diploid species found throughout the world. These variable sequences and their rate of evolutionary change provide numerous characters (both nucleotide substitutions and indels) at this taxonomic level in *Isoëtes*.

The tree resulting from the combination of ITS and spacer data are of interest biogeographically (Fig. 1). The well supported Old World/California clade consists of species with Eurasian affinities. The inclusion of the two Californian species, *I. nutallii* and *I. orcuttii*, within this clade suggests that this group of *Isoëtes* may have previously had a distribution throughout Asia and into North America. *Isoëtes abyssinica* is sister to the two Spanish species, *I. longissima* and *I. velata*, indicating a strong affinity between at least some North African and European species. These results also support at least two origins for the North American species of *Isoëtes*, those with Eurasian affinities as described above and the many species found in the North American species complex. Current work is now underway which will greatly increase our sampling worldwide, further testing the above biogeographical hypotheses.

The lack of variation in the North American species complex using two

TABLE 2. ITS nucleotide additivity in *Isoëtes* × *eatonii* and *I. riparia* at sites where the parental species, *I. englemannii* and *I. echinospora*, differ.

	Sites and Nucleotides									
Taxon	39	83	274	314	319	321	544	627	644	679
I. engelmannii	A	C	T	G	G	A	T	C	G	G
I. echinospora	T	T	C	C	T	T	G	T	A	T
I. Xeatonii	A/T	C/T	C/T	C/G	G/T	A/T	G/T	C/T	A/G	G/T
I. riparia	T	C/T	C	C/G	G/T	A/T	G/T	C/T	A/G	G/T

different combined data sets, ITS/spacer (Fig. 1) and *LFY*/ITS (Fig. 2), suggests a rapid radiation in the North American species complex. However, the *LFY*/ITS tree does resolve a sister group relationship between *I. hawaiiensis* and three populations of *I. echinospora* (8 substitutions, 2 indels, bootstrap = 100%), suggesting that a founder population of *I. echinospora* made its way to Hawaii from the boreal regions of the Northern Hemisphere, possibly by means of migrating waterfowl. *Isoëtes hawaiiensis*, while exhibiting considerable morphological divergence from *I. echinospora,* is virtually identical in terms of molecular data (two character differences), suggesting that the event occurred relatively recently.

SPECIES DELIMITATION WITHIN THE NORTH AMERICAN SPECIES COMPLEX.—A persistent problem in molecular plant systematics has been the lack of DNA sequence regions variable enough to be used at the species and population level. The second *LFY* intron is proving invaluable in this regard. In *Isoëtes, LFY* is at least four times more variable than ITS, which is generally regarded as one of the more variable sequence regions available for systematic studies.

The species identity found in the *LFY*/ITS tree (Fig.2) is largely based on *LFY* data due to the greater variation found in this region. *LFY* provides numerous characters, both substitutions and indels, which can serve to delimit and identify species. For example, our results indicate that widely separated populations of *I. engelmannii* (Michigan, Missouri, and Virginia) and *I. echinospora* (Maine, Montana, and Wisconsin) are virtually identical.

In contrast, the *I. melanopoda* complex contains at least one cryptic species. The population of *I. melanopoda* from Arkansas is very different molecularly from the two populations from Louisiana and Mississippi, coming out within a moderately supported clade (bootstrap = 71%) consisting of *I. lithophila* from Texas and *I. tegetiformans* from Georgia. Work is currently underway to further test species limits for other *Isoëtes* taxa.

DETERMINING THE ORIGIN OF ALLOPOLYPLOID SPECIES.—Additive banding and sequence misalignment in ITS data from hybrids and allopolyploids are valuable tools in the confirmation of interspecific hybrids and the determination of their parental origins. For the most part, the additive banding found in the hybrid *I.* ×*eatonii* and the allotetraploid *I. riparia* confirm that their progenitors were *I. echinospora* and *I. engelmannii* (Table 2). Two of the sites (39, 274) do not show additivity in *I. riparia*, but do possess nucleotides at these

sites that are found in one of the putative parents. This loss of additivity may be due to the effects of concerted evolution, mutation events, or sequence differences in one of the diploid parents of *I. riparia* not detected with our sampling. We are currently studying hybrid origins of the above and other allopolyploids using *LFY* data. Because many more substitutions and indels occur with *LFY* data, cloning is being employed to separate the two parental copies. Our preliminary data for *LFY* also support the above findings for the origins of *I. riparia*.

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